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Study of oligogalacturonides-triggered nitric oxide (NO) production provokes new questioning about the origin of NO biosynthesis in plants

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We investigated the production and function of nitric oxide (NO) in *Arabidopsis thaliana* leaf discs as well as whole plants elicited by oligogalacturonides (OGs). Using genetic, biochemical and pharmacological approaches, we provided evidence that OGs induced a Nitrate Reductase (NR)-dependent NO production together with an increased NR activity and NR transcripts accumulation. In addition, NO production was sensitive to the mammalian NOS inhibitor L-NAME. Intriguingly, L-NAME impaired OG-induced NR activity and did not further affect the remaining OG-induced NO production in the *nia1nia2* mutant. These data suggest that the l-arginine and NR pathways, co-involved in NO production, do not work independently. Taking account these new data, we propose scenarios to explain NO production in response to biotic stress.

Many studies indicate that NO acts as a signaling compound in plants, particularly in physio-pathological context where NO production was reported to be a conserved event in plant-pathogen interaction.[1-3] However, one major unresolved issue concerns the enzymatic sources of NO: although many efforts have been made, the mechanisms underlying NO synthesis in plants remain a black-box. This limitation severely hinders rapid progress in our understanding of NO physiological functions in plants. In animals, NO is mainly synthesized from l-arginine and oxygen by nitric oxide synthase (NOS). In contrast, several enzymatic sources of NO have been proposed for NO synthesis in plants. To date, at least seven pathways of NO synthesis have been identified (reviewed in ref. 4). The two most documented synthesis pathways are (i) a l-arginine-dependent pathway, sensitive to mammalian NOS inhibitors, involving NOS-like activities although there is no obvious homologs of mammalian NOS in the land plant genomes sequenced so far[5] and (ii) a nitrite-dependent pathway involving the Nitrate Reductase enzyme that could reduce nitrite to NO both in vitro and in vivo.[6]

In a recent publication,[7] we characterized oligogalacturonides (OGs)-induced NO production in *Arabidopsis thaliana* plants and investigated its incidence in defense responses. The OGs, structural components of the plant cell wall, are considered as endogenous elicitors of plant defense and represent a valuable tool to analyze the NO-related mechanisms involved in plant-pathogen interaction. In this work, we showed that OGs treatment of leaf discs triggered an intracellular accumulation of NO using two different fluorescent indicators, the DAF-2 and the CuFL probes.[8,9] Analysis of the signaling pathway involving NO showed that its production is Ca^{2+}-dependent and modulates AtRBOHD-mediated ROS production. We also provided evidence that NO, as well as two identified target genes encoding the anionic peroxidase (PER4) and a β-1,3-glucanase, contributes to the OG-triggered immunity against *Botrytis cinerea*. Taken together, our data reinforce the concept that NO is a key mediator of plant defense responses.

Beside these results deciphering the role of NO in oligogalacturonides-triggered

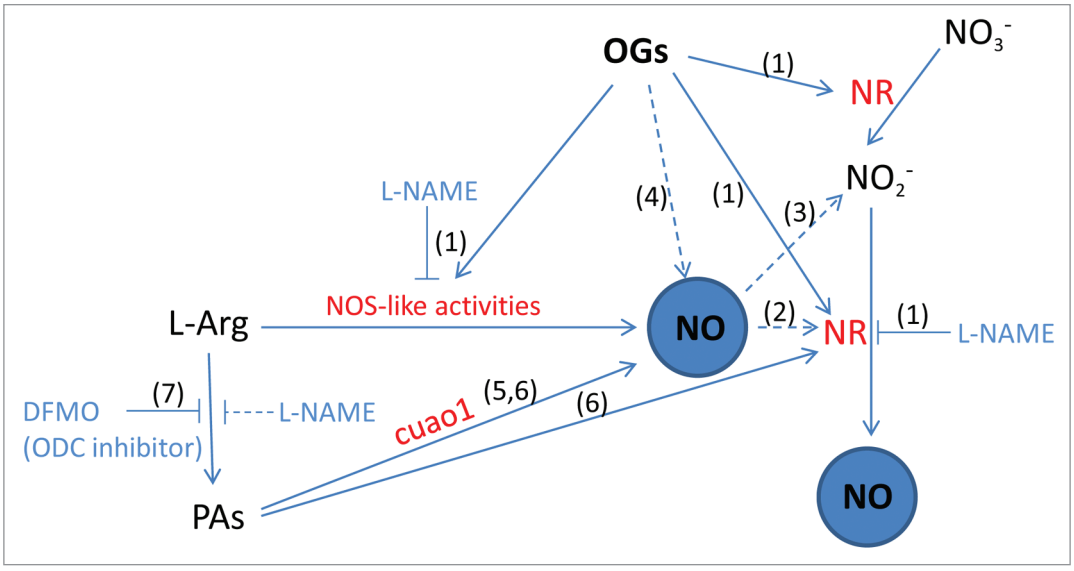

Figure 1. Enzymatic sources of NO production in response to OGs. OGs induced a NR-dependent NO production together with an increased NR activity. This NO production was sensitive to the mammalian NOS inhibitor L-NAME suggesting that OGs-induced NO synthesis might also involve a l-arginine-dependent process. L-NAME impaired OG-induced NR activity[7] (1). NO produced by l-arginine-dependent pathway could modulate NR activity[14] (2) or could be oxidized to nitrite, thus providing substrate for NR-triggered NO synthesis (3). These data and hypotheses are consistent with the involvement of an alternative and unknown route for NO production (4). PAs triggered NO production suggesting the involvement of PA metabolism, particularly one copper aminooxidase1 (cuao1), in NO production in plants[11,12] (5). Moreover, PAs modulated NR activity by increasing NO and by affecting 14-3-3 proteins interaction with NR[13] (6). Accordingly, DFMO, inhibitor of PA synthesis, reduced NO production (7) (Rasul S, et al. unpublished results).

immunity and resistance to *Botrytis cinerea* in *A. thaliana*, we investigated the enzymatic sources producing NO. Our data provide several lines of evidence implicating NR a major source for this NO production. First, OG-induced NO production was reduced by 50% in the NR deficient double mutant *nia1nia2* and was partly suppressed by the NR inhibitor tungstate. Second, the NR-dependent NO production was correlated with enhanced NR activity and upregulation of *NIA1* and *NIA2* gene expression. Third, l-arginine supply did not restore OG-induced NO production in the *nia1nia2* mutant, excluding the possibility that the lower level of NO observed in *nia1nia2* in response to OGs is related to an l-arginine deficiency in the leaves as previously reported in reference 10. In addition to NR, we provided data suggesting that OGs-induced NO synthesis might also involve a l-arginine-depend process (approx 50%). Indeed, the production of NO induced by OGs was reduced by 50% by L-NAME, a mammalian NOS inhibitor previously shown to suppress NOS-like activities in plants. Interestingly, the Ca^{2+} channel blocker La^{3+}, did not reduce in vivo OG-induced NR activity and the *nia1nia2* mutant behaves like wild type plants in terms of OG-induced ROS generation. These data reinforce the hypothesis that NO production might involve at least two enzymatic sources, NO resulting from the l-arginine-dependent pathway being involved in the control of the oxidative burst and Ca^{2+}-dependent in contrast to NR dependent NO production.

Relationships between these two pathways for NO synthesis were further investigated. We observed that L-NAME did not affect the remaining OG-induced NO production in the *nia1nia2* mutant. Surprisingly, we observed that L-NAME inhibits OG-induced NR activity. These data suggest that the l-arginine and NR pathways, co-involved in NO production, do not work independently. However, one must be cautious on interpretation based on L-NAME. Indeed, the molecular target(s) of L-NAME are unknown in plants and we should not rule out the possibility that L-NAME treatment also triggers metabolic disorders impacting NO synthesis through unspecific and unknown processes. For example, beside NOS, L-NAME could also affect the activities of other l-arginine metabolizing enzyme such as enzymes of the polyamine (PA) metabolism pathways expected to be involved in NO production.[11,12] Very recently, Rosales and colleagues[13] showed that Spd and Spm significantly modulate NR activity in a NO-dependent manner or by modifying the interaction between NR and 14.3.3 proteins evoking a link between NO production, NR activity and PA metabolism.

According to our study[7] and previously published works, we propose different scenarios to explain OG-induced NO production (**Fig. 1**). In a first scenario, based on the likely poor specificity of L-NAME, we propose that NR is the main source for NO in response to OGs and L-NAME effect on NO synthesis and NR activity could reflect unspecific action of the NOS inhibitor. In a second scenario, based on studies showing that L-NAME inhibits NOS-like activities in plants, we suggest that L-NAME-sensitive NO production affect NR-dependent NO production: NO can stimulate NR activity at the post-translational level though a direct interaction or, alternatively, by affecting the activity of proteins involved in NR

regulation. However we can assume that part of the NO produced by l-arginine-dependent pathway could be oxidized to nitrite, thus providing substrate for NR-triggered NO synthesis. These scenarios are consistent with the involvement of PAs or/and an alternative and unknown route to those pathways. The identification of the enzymatic source producing NO from l-arginine (or L-NAME sensitive) and a detailed analysis of the molecular mechanism underlying L-NAME effect on nitrogen metabolism remain two essential issues to be clarified.

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